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14. ABSTRACT

We have completed proposed research in the First Year Task (i) both antioxidants, N-acetylcysteine and ebselen, overexpression of ROS lowering genes, such as, catalase or mtSOD; and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA and a part of Second Year Task (iii) estrogen-induced cell transformation experiments determined by: (a) Foci Formation, (b) Anchorage-independent (soft agar) cell growth showed that estrogen-induced conversion of normal cells to transformed cells is inhibited by treatment with N-acetylcysteine and ebselen, overexpression of MnSOD or catalase; or mtTFA silencing. Our study revealed: i) Inhibition of ROS formation or detoxification of ROS prevented estrogen-induced DNA synthesis. ii). Both antoxidant treatment and detoxification of ROS prevented E2-induced expression of cyclin D1 and pcna. iii) E2 dependent anchorage-independent growth of MCF-10A cells is dependent on ROS and is prevented by overexpression of MnSOD, catalase, or mtTFA silencing. These findings suggest that, in addition to the receptor activity of estrogens, other factor(s) are involved in the stimulation of cell growth by estrogen. Our data suggest that estrogen regulates cell cycle genes through ROS. In addition to, existing antiestrogens and anti-aromatse inhibitors, validation of our novel concept that defines estrogen-induced mtROS-dependent signaling pathways that distinguish between cell transformation and tumor cell growth will help to develop antioxidant-based drug or gene therapies for the prevention and treatment of estrogen-dependent breast cancer.

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Table of Contents

Responses to the reviewer's comments

I have revised the report and included data, figures, tables with legends to substantiate the finding in the text.

The assumption that " the PI has made substantial changes in the Statement of the Work and has NOT approved" are not correct. As I described previously that we had proposed to complete the proposed research in two years at the time of submission of proposal. However, during the technical aspects of the award negotiations and the regulatory approval process , we were asked to make it to three years (see e-mail from Monica J. Pileggi Contract Specialist, dated February 21, 2004 in the appendix). Accordingly, proposed two year's budget was changed to three years, because we were informed that "**The Grants Officer Representative has recommended that you revise the budget from two years to three years. This will allow the PI to complete the project in a reasonable time frame"** (see e-mail from Monica J. Pileggi Contract Specialist, dated February 21, 2004 in the appendix)**.** We complied to this request. However, we were not asked to change the Statement of Work. The original Statement of Work to be completed in two years will be completed in three years.

Additionally, we were asked to address the issue from our Summary Statement during the technical aspects of the award negotiations and the regulatory approval process (see e-mail from the Dr. Donna M. Kimbark, dated January 27, 2006). I submitted my responses addressing the issue from the Summary Statement on February 24 to Dr. Kimbark (see the copy of my e-mail and my responses in the appendix) and later the same document was forwarded through the FIU"s Office of Sponsored Research on March 1, 2006.

The Revised Report

Introduction: Recently, we reported that mitochondria are significant targets of estrogen (Felty and Roy, 2005). There is considerable evidence, both experimental and epidemiologic, that estrogens play a role in carcinogenesis, however, these effects cannot be fully accounted for by the mitogenic effects induced by estrogen stimulation of their receptors (ER) (Roy et al, 2004). Both nongenomic estrogen-induced reactive oxygen and nitrogen species (RO/NS) and direct transcriptional ER effects are required to promote DNA synthesis. In this application we have proposed to investigate the role of estrogen-induced mitochondrial (mt) oxidant signaling pathways in the *in vivo* progression of breast cancer as a new line of research that may lead to the discovery of novel antioxidant-based drugs or new antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer. We proposed to examine an untested and highly innovative concept in estrogen-induced carcinogenesis research, *i.e.*, estrogen-induced mitochondrial oxidants are involved in the promotion/progression of breast cancer through modulating signaling that controls the early G1 stage of the cell cycle.

We have completed proposed research in the original First Year Task (i) both antioxidants, N-acetylcysteine and ebselen, overexpression of ROS lowering genes, such as, catalase or PrxIII; and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA by real-time RT-PCR, the rate of DNA synthesis by BrDu incorporation, and different phases of cell cycle by

flow cytometry and the most of the part of Task (ii) by determining the morphology and behaviors of cells that over-express mtTRX2, mtSOD, mtPrxIII, catalase or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to estrogen. Findings are described in detail below:

1. Treatment with antioxidant as well as overexpression of catalase and MnSOD prevent estrogen-induced DNA synthesis: Recently, we have shown that physiological concentrations of E2 result in the rapid production of ROS in epithelial cells (Felty et al. 2005). In order to verify that our cells responding to E2 exposure in terms of ROS production, first we measured the formation of ROS upon 17 beta-estradiol exposure (E2). MCF7 cells were grown in 96-well plates for 2 days in 10% FBS DMEM/F12 and serum starved 2 days prior to addition of E2 for 18 h-48 h. As expected estradiol induced oxidants production in MCF-7 cells in dose dependent manner (Fig. 1).

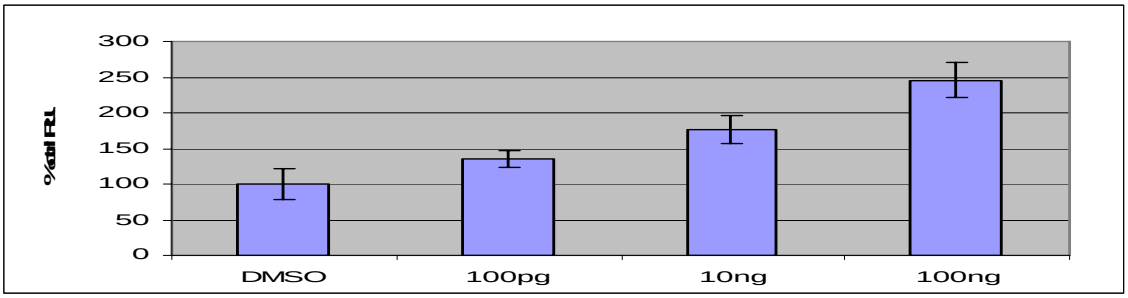


Figure 1 Estrogen induces ROS formation in MCF-7 cells in dose dependent manner as assayed by DCFDA oxidation. MCF-7 cells (5000 cells/well) were growth synchronized by culturing it in growth media w/o serum for 48hrs post seeding. DCFDA were loaded with 10uM for 20mins after which cells were exposed to various concentration of E2 and emissions read at 10mins post treatment on fluorescence plate reader. The level of ROS is expressed as relative fluorescence unit and vehicle (DMSO) treated value was considered 100%.

Similarly, we characterized the effect of different concentrations of E2 on cell proliferation. The incorporation of BrDu showed that E2 induced cell proliferation in MCF-7 cells in a dose dependent manner. This is in agreement with previous findings (Foster et al., 2001).

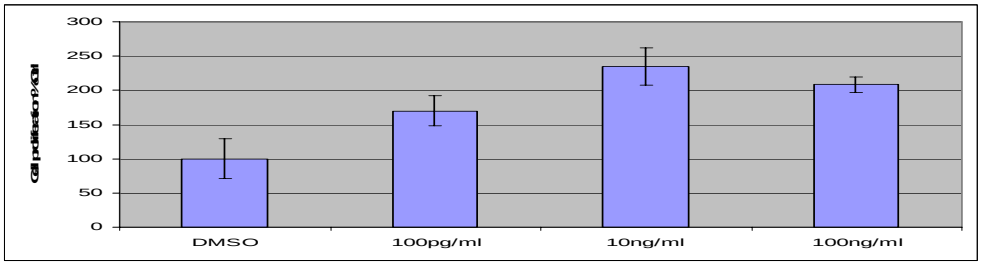


Figure 2. Estrogen induced BrdU incorporation in MCF-7 is dose dependent. MCF-7 cells were growth synchronized by culturing cells in growth media without serum for 48hrs post seeding. Cells were then exposed to various concentration of estrogens for 24hrs after which BrdU incorporation were measured and compared against vehicle.

Antioxidants reduce E2-induced cell growth. Growth factors are known to stimulate a rapid increase of intracellular ROS that participate in cell signaling pathways involved in growth. Recently, we have shown that physiological concentrations of E2 result in the rapid production of ROS in epithelial cells (Felty et al. 2004), however, the biological role of these oxidants in the control of E2-induced cell growth is not clear. Therefore, we evaluated the effect that antioxidants had on E2-induced growth of epithelial cells. As shown in Fig. 3A, NAC reduced E2-induced cell growth to the level of the control while the antioxidant enzyme catalase significantly inhibited E2-induced cell growth by as much as 50% after a 72 h treatment. The reduction of E2-induced cell growth observed with NAC and catalase treatment was confirmed by the SRB assay (Fig. 3B). The SRB assay also demonstrated that the glutathione peroxidase mimic, ebselen, effectively reduced E2-induced cell growth to the level of the control. These findings indirectly demonstrate that E2-induced oxidants support the growth of epithelial cells.

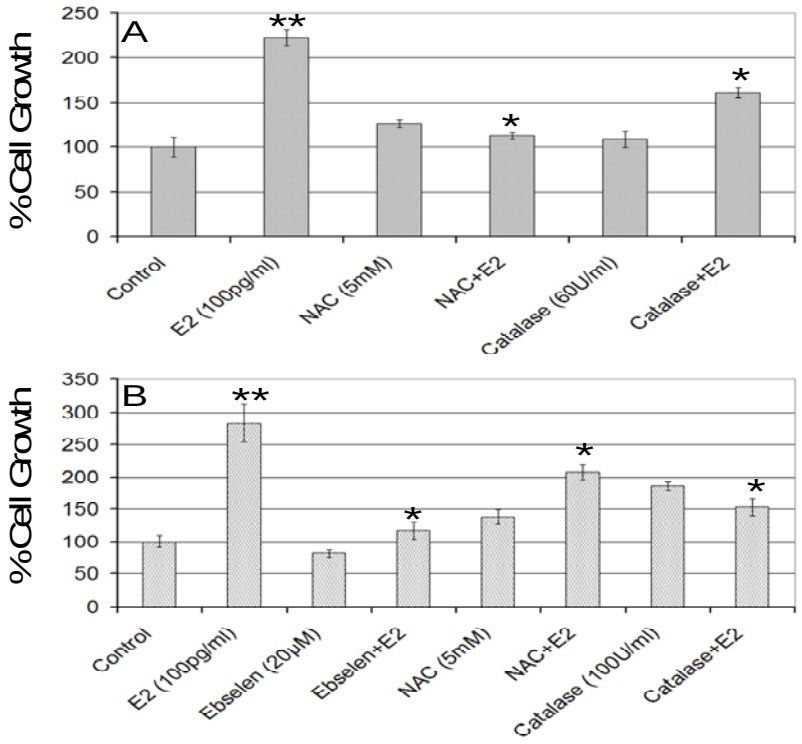


Fig. 3. Antioxidants reduce E2-induced breast cancer cell growth. MCF7 were cultured in T-25 flasks for 1 day in 10% FBS DMEM/F12 and serum starved for 2 days prior to treatment with E2 for 72 h unless specified otherwise. (A) Cell counts of MCF7 cells pretreated NAC and catalase for 2h prior to addition of E2. Results are expressed as means ± SD of three separate experiments with control set as 100% cell growth. (**) Indicates treatment significantly different from control. (*) Indicates treatment significantly different from E2. (P<0.05). (B) Verification of cell growth by SRB assay. MCF7 cells were seeded in a 24-well plate ($4x10^4$ cells/well). Results are expressed as mean OD ± SD of three separate experiments with control set as 100% cell growth. (**) Indicates treatment significantly different from control. (*) Indicates treatment significantly different from E2. (P<0.05).

<u>Antioxidants and ROS detoxifying enzume suppress E2-induced DNA synthesis.</u> In order to confirm the previous data on the effect of antioxidants on cell proliferation, we tested the influence of antioxidants on E2-induced DNA synthesis. E2-induced DNA synthesis, as evaluated by BrdUrd incorporation, was significantly decreased 50% by both ebselen and NAC treatments at 18 h (Fig. 4A). This was also the case for the antioxidant enzyme catalase. The suppression of DNA synthesis by antioxidants further supports the hypothesis that E2-induced ROS modulates the process of cell proliferation.

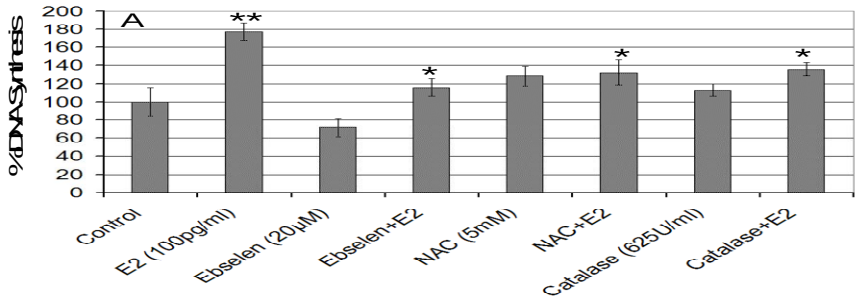


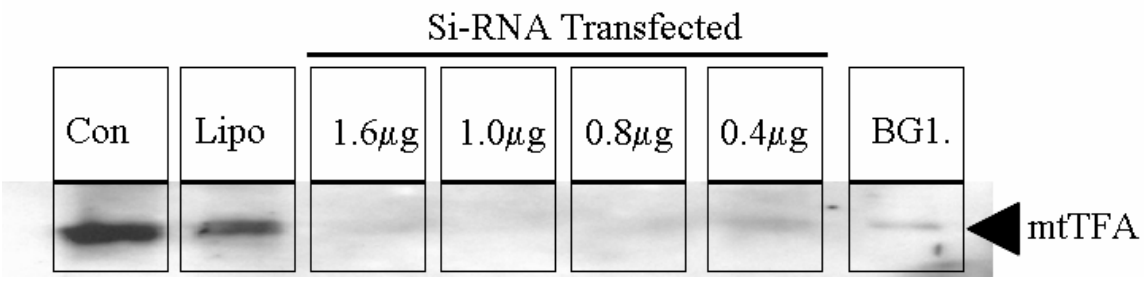
Fig. 4. Antioxidants reduce E2-induced DNA synthesis in breast cancer cells. MCF7 were grown in 96-well plates for 2 days in 10% FBS DMEM/F12 and serum starved 2 days prior to addition of E2 for 18 h-48 h unless specified otherwise. (A) BrdUrd incorporation assay on MCF7 cells. Antioxidants ebselen, NAC, and catalase were pretreated for 2 h prior to the addition of E2. Colorimetric BrdUrd incorporation was measured at 450 nm with a plate reader. Results are expressed as mean OD ± SD of three separate experiments with control set as 100% DNA synthesis. (**) Indicates treatment significantly different from control. (*) Indicates treatment significantly different from E2. (P<0.05).

2. Mitochondrial biogenesis blocker prevents E2-induced G1/S transition of G0 arrested cell. Since mitochondria are a major source of E2-induced ROS in breast cancer cells, we used different approache to test whether mitochondrial ROS play a role in E2-induced DNA synthesis. Recently, the regulation of G1 to S phase transition in fibroblast cells was reported to depend on the intracellular redox state (Menon et al. 2003). Since our data suggested that DNA synthesis is controlled by E2-induced ROS, we further evaluated the fate of E2 treated cells within the cell cycle by controlling mitochondrial biogenesis via silencing the expression of mtTFA. Mitochondrial transcription factor A (mtTFA) is a key regulator of the mtDNA copy number in mammals. MtTFA controls mt DNA copy number and is essential for mitochondrial biogenesis [Larson et. Al., 1998]. MtTFA translocates into mitochondria and initiates the transcription and replication of the mt genome. To determine the involvement of mTFA in estrogen-induced cell proliferation, we silenced mtTFA expression in MCF7 cells. siRNAs targeting mtTFA mRNA sequence were designed, and a standard scramble siRNA was used as control. . Our insert sequence for siRNA expression vector containing siRNA target sequence for mitochondrial transcription factor A (mtTFA):GATCCCAAGGGGGAGCGCAGTCGACTTCAAGA

GAGTCGA CTGCGCTCCCCCTTTTTTTTCCAACTCGAG.

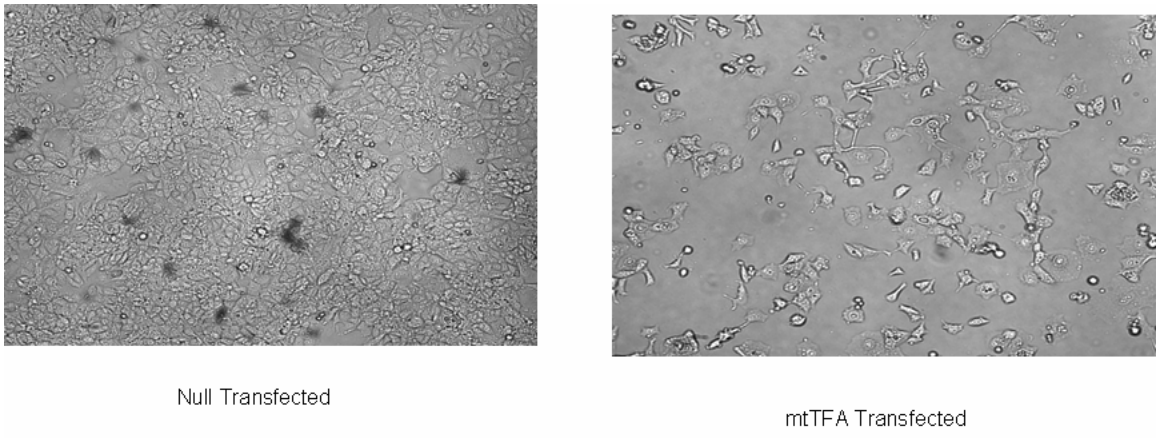
Cells (150,000) were seeded in 6 well plate, grown overnight in DMEM F12 medium, transfected with different amount of pSilencer 1.0-U6-mtTFA siRNA expression vector (Ambion). Transfected cells grown for 36 hrs, cell lysates from these cells were prepared and t*he expression of mtTFA protein was detected by Western blotting (Co= Control, Lip = Lipofectamine alone, 0.4 u-1.6 u are different concentrations of mtTFA SiRNA).* Transfection with siRNA induced a significant down-regulation of mtTFA at potein level as shown below in Figure 5.

Fig. 5



Silencing of mtTFA alone resulted in significant inhibition of growth of MCF-7 cells shown in Figure 6. The viability of mtFTA silenced cells were so low, we could not meaure its effect on E2-induced cell growth. Figure 6 shown below represents the 10 X pictures of scramble SiRNA and mtTFA SiRNA after 56 hrs of transfection.

Fig. 6



In order overcome this challenge, we put the SiRNA construct in tet off system. The inhibition of the expression of mtTFA was controlled by the addition of different amounts of antibiotics. growth inhibitory effects To examine the effect of mtTFA on estrogen-induced for proliferation, mtTFA SiRNA containing cells were harvested from six-well plates 48 h posttransfection and seeded (1500 cells per 0.2 mL) in triplicate in 96-well plates. Its subsequent effects on proliferation of MCF-7 cells were determined by BrDU incorporation. For cell cycle progression analysis, cells were synchronized by serum starvation for 24 h and induced to re-enter the cell cycle by E2 (100 pg/ml) treatment in both wild type and mtTFA silenced MCF-7 cells. Fluorescence-activated cell sorting (FACS) analysis of DNA content was performed on cells stained with propidium iodide. FACS results are summarized in Table 1.

Table I. The effect of mitochondrial blocker on E2-induced cell growth.

For BrDU incorporation, MCF7 wild type and mtTFA silenced cells (2500/well) were grown in 96-well plates for 2 days in 10% FBS DMEM/F12 and serum starved 2 days prior to addition of E2 (100 pg/ml) for 18 h-48 h unless specified otherwise. Colorimetric BrdUrd incorporation was measured at 450 nm with a plate reader. Results are expressed as mean OD ± SD of three separate experiments with control set as 100% DNA synthesis.

For flow cytometry both wild type MCF7 and mtTFA silenced MCF-7 cells were seeded (5x105 cells) in T-25 flasks grown 1 d in 10%FBS DMEM/F12, serum starved 1 d in DMEM/F12, and treated with E2 (100pg/ml) for 24h. The distribution of cells in the cell cycle was determined by flow cytometry using propidium iodide-stained nuclei. Fluorescence-activated cell sorter (FACS) data are presented as representative histograms from three separate experiments. Data were analyzed by ModFitLt® V2.0 cell cycle kinetics.

	% BrDu Incoporation	% DNA		
		G0/G1	S	G2/M
Wild Type mtTFA cells				
Control	100%	83.2	8.8	8
E2	180%	74.6	18.1	7.3
mtTFA silenced cells				
Scrambled SiRNA	92%	84.9	9	6.1
E2	93%	84	6.8	9.1

mtTFA siRNA transfection inhibited estrogen-induced proliferation of MCF-7 cells which is evident from the lower incorporation of BrDU in SiRNA treated cells compared to wild type cells in the presence of E2. We observed similar results by flow cytometery. In E2 treated MCF7 cells, the percentage of DNA content in S phase was 18% while this decreased to 6.8% in mtTFA silenced MCF7 after 24 h (Table I). %. The FACS data not only confirms the results shown by the BrdUrd assay, it also shows that impairment of mitochondrial biogenesis prevents E2-induced entry of MCF7 cells into the S phase by arresting them in the G0/G1 phase.

E2-induced cell growth is inhibited by the overexpression catalase, Trx2 or mt PrxIII: The major mitochondrial antioxidant system is made of MnSOD on the one hand, and of peroxiredoxin III, mitochondrial thioredoxin (Trx2) and mitochondrial thioredoxin reductase (TrxR2) on the other hand. Peroxiredoxins (Prxs) are a recently characterized group of thiol-containing proteins with efficient antioxidant capacity, capable of consuming hydrogen peroxide in living cells. Altogether six distinct Prxs have been characterized in mammalian tissues and Prxs III and V are localized in mitochondria [43]. With the exception of thioredoxin reductase, all these proteins are induced by oxidative stress. A function of the mitochondrial thioredoxin system is as electron donor for a mitochondrial peroxiredoxin, an enzyme that detoxifies the

hydrogen peroxide generated by mitochondrial metabolism. We proposed to demonstrate that E2-induced cell growth by RO/NS signaling is inhibited in the presence of overexpressing Trx2 or mt PrxIII vectors, which are considered to lessen the level of mt oxidants.

To examine the effect of Trx2 or PrxIII on estrogen-induced for proliferation, MCF-7 cells were transfected with catalase, Trx2 or mt PrxIII mammalian vectors and harvested from six-well plates 48 h posttransfection and seeded (1500 cells per 0.2 mL) in triplicate in 96-well plates. Its subsequent effects on proliferation of MCF-7 cells were determined by BrDU incorporation. E2-induced DNA synthesis was drastically reduced in Trx2 and PrxIII overexpressing cells, which scavenges ROS. Adenvirus construct containing catalase that lowers oxidant production also decreased E2-induced DNA synthesis to control level, however, MnSOD failed to produce growth inhibitory effects on E2 exposed cells. Our findings suggest that oxidant signaling from the mitochondria to the nucleus supports E2-induced DNA synthesis.

Table II. The effect of overexpression of Trx2 and PrxIII on E2-induced cell growth.

For BrDU incorporation, MCF7 wild type and catalase, MnSOD, Trx2 and PrxIII overexpressing cells (2500/well) were grown in 96-well plates for 2 days in 10% FBS DMEM/F12 and serum starved 2 days prior to addition of E2 (100 pg/ml) for 18 h-48 h unless specified otherwise. Colorimetric BrdUrd incorporation was measured at 450 nm with a plate reader. Results are expressed as mean OD ± SD of three separate experiments with control set as 100% DNA synthesis.

	% BrDu Incoporation
Wild Type cells	100%
MnSOD overexpressing cells	80%
Catalase overexpressing cells	95%
Trx2 overexpressing cells	101%
PrxIII overexpressing cells	105%
E2	185%
Catalase overexpression + E2	115%
MnSOD overexpression + E2	190%
Trx2 overexpression + E2	145%
PrxIII overexpressing cells	141%

Both antoxidant treatment and dextoxification of ROS prevented E2-induced expression of cyclin D1 and pcna, markers of cell proliferation detected by Real time PCR. We first determined whether E2-induced expression of pcna was modulated by oxidants. MCF7 cells were pretreated for 2 h with the antioxidants ebselen (20 μM) and NAC (10 mM) followed by a 18 h E2 treatment. Real-time PCR analysis showed a decrease in the level of pcna by both antioxidants when compared to E2 treatment alone (Table III, lower panel). In addition, overexpression of catalase and MnSOD known to detoxify ROS decreased the level of E2-induced pcna (Table III, upper panel).

Next, we evaluated whether the oxidant-dependent expression of cyclin D1 was a function of mitochondrial ROS. As shown in Table III, middle panel, real-time PCR analysis showed a decrease in the level of cyclin D1 expression by overexpression of catalase but not by mnSOD when compared to E2 treatment alone.

Sample	Detector	Avg Ct	dCt	ddCt	fold increase	relative ma
dmso 18 hrs	pcna	21.735	7.456	0	1	10
e2 100pg	pcna	21.654	6.991	0.47	1.38	14
MnSOD+dmso	pcna	22.354	7.79	0	1	10
MnSOD+E2	pcna	22.413	7.925	-0.14	0.91	9
catalase+dmso	pcna	21.588	7.412	0	1	10
catalase+E2	pcna	22.075	7.566	-0.15	0.9	9

Sample	Detector	Avg Ct	dCt	ddCt	fold increase	relative ma
dmso 3 hrs	cyclin D1	29.362	15.083	0	1	10
e2 100pg	cyclin D1	29.121	14.458	0.62	1.54	15
MnSOD+dmso	cyclin D1	30.103	15.539	0	1	10
MnSOD+E2	cyclin D1	29.708	15.221	0.32	1.25	13
catalase+dmso	cyclin D1	28.531	14.355	0	1	10
catalase+E2	cyclin D1	30.045	15.536	-1.18	0.44	4

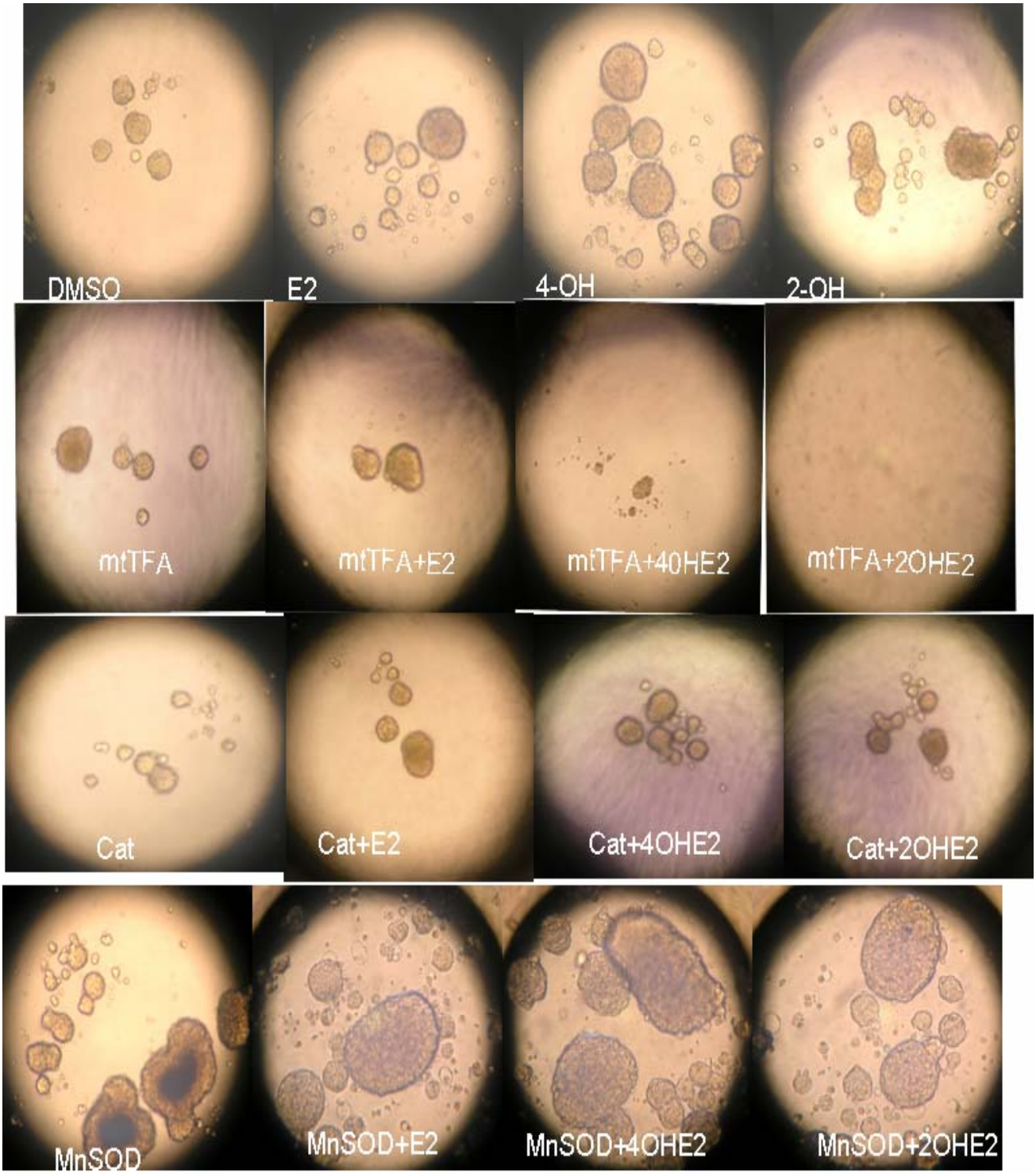
Sample	Detector	Avg Ct	dCt	ddCt	fold increase	ve mRNA cc
dmso 18 hrs	pcna	35.139	20.86	0	1	10
e2 100pg	pcna	35.089	20.426	0.43	1.35	14
Ebselen+dmso	pcna	34.976	20.412	0	1	10
Ebselen+E2	pcna	34.732	20.244	0.17	1.12	11
NAC+dmso	pcna	32.927	18.751	0	1	10
NAC+E2	pcna	33.463	18.954	-0.2	0.87	9

Table III: Real-time PCR data of cyclin D1 expression after 3 h E2 treatment. MCF7 cells ($5x10^5$) were grown 1 day, serum starved 2 days, prior to E2 treatment. For pcna cells were treated with E2 for 18 hrs. Pretreatment with ebselen (20 μM) and NAC (10 mM) was followed by 3h E2 treatment.

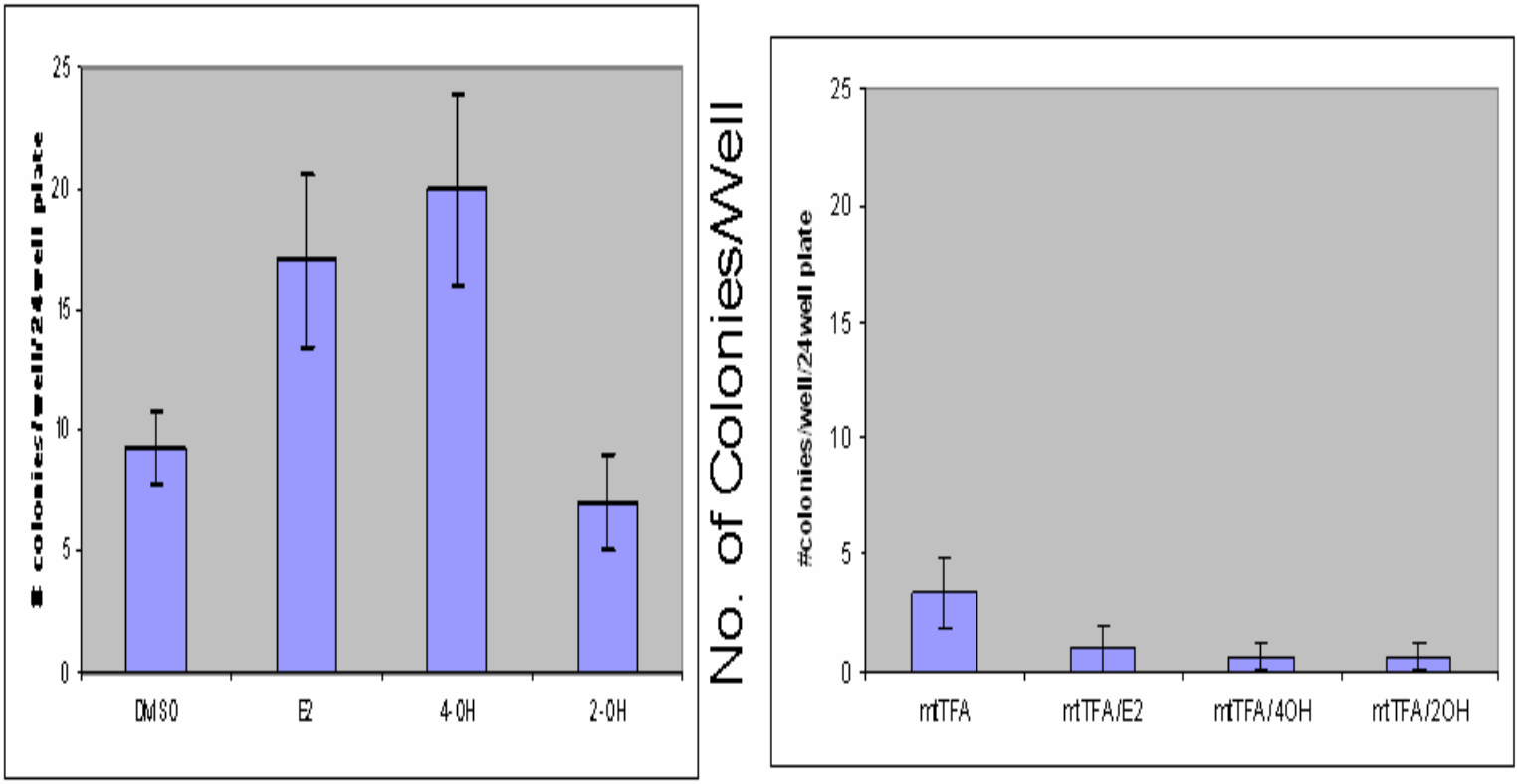
4. The E2-induced in vitro tumorigenicity of breast cancer cells is inhibited by the overexpression of catalase and by silencing of mtTFA: Since our major intention of this proposed research is to discover novel antioxidant-based drugs or new antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer, we tested the effects of overexpression of catalase and MnSOD as well as silencing of mtTFA on in vitro tumorigenicity of breast cancer cells. The ability of cells to proliferate in the absence of adhesion to the extracellular matrix (ECM) or anchorage-independent growth is one of the hallmarks of transformed cells and is also the best in vitro correlate of tumorigenicity. For this study, we used CHEMICON®'s Cell Transformation Detection Assay (Chemicon Int, CA), an anchorage-independent growth (AIG) assay in soft agar. After 1 day, the cells are treated with 10 ng/ml of E2 or 4-OH-E2. The medium 2 is renewed every 3 days. At the end of 2 weeks, the medium was removed and colonies of cells are stained with crystal voilet. Colony efficiency was determined by a count of the number of colonies >63 μm in diameter and expressed as a percentage of the original number of viable cells after 24 h of plating. Our study revealed that in the anchorage independent growth (AIG) assay as shown above, both E2 and 4-OH-E2 were able to induce AIG positive colony formation. In cells overexpressed with Adenvirus construct containing catalase that lowers oxidant production as well as in mtTFA silenced cells using

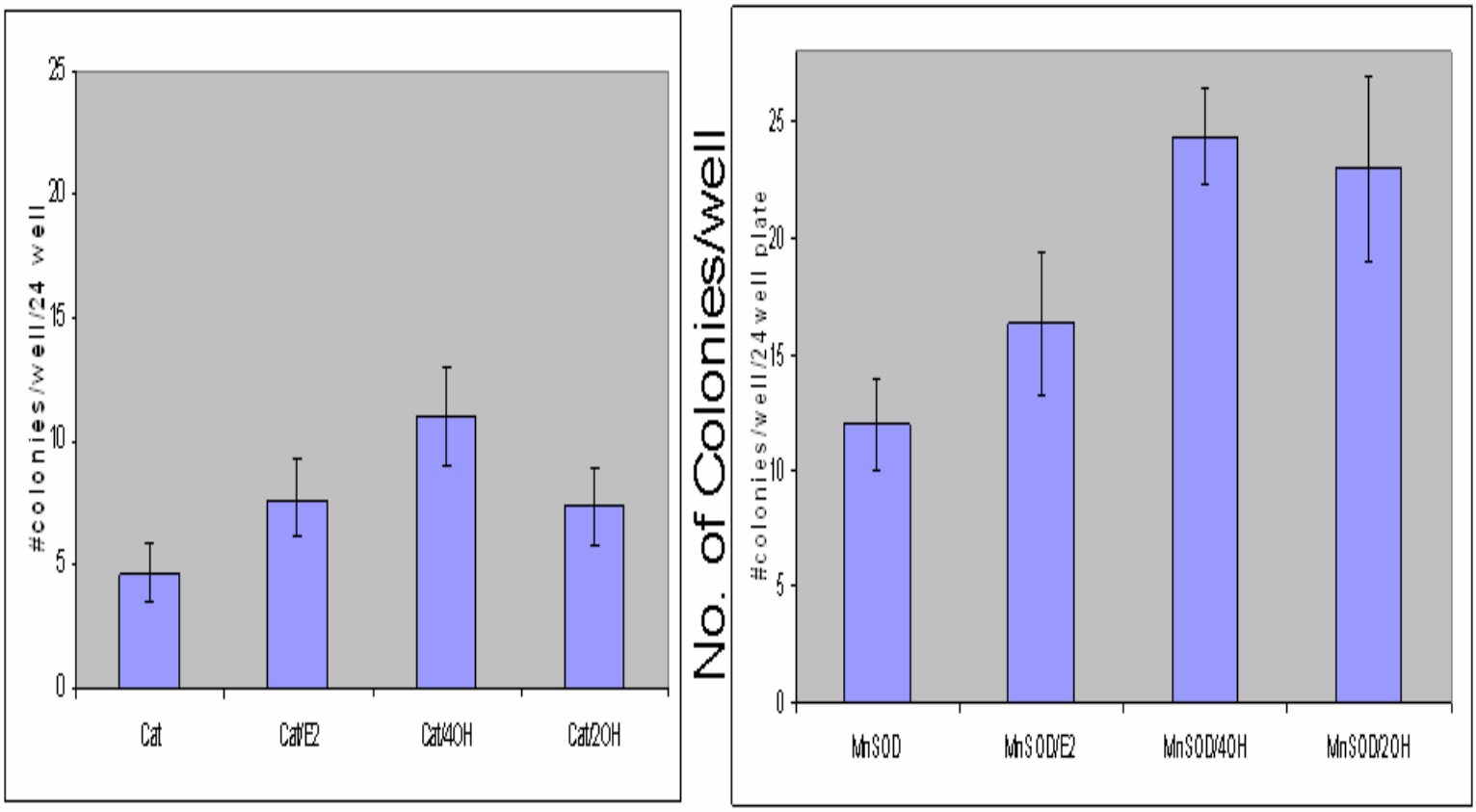
their SIRNA, E2 was not able to produce any colony (Figure 7A&B). Both antioxidants ebselen and N-acetylcysteine produced similar effects. MnSOD, which dismutates superoxide radicals to hydrogen peroxide, did not inhibit estrogen-induced colony formation of MCF-7 cells. We are currently working to analyze the expression of different amounts of MnSOD on cell growth. Based on these data it appears that E2 dependent colony formation rate of MCF-7 cells is dependent on ROS or mitochondrial signaling.

7A. Representative pictures of estrogen-dependent colony formation in the presence of various treatment.



7B. Inhibition of estrogen- dependent colony formation by overexpression of catalase and silencing of mtTFA. Each value is an average of 3 experiments +std. dev.





5. Summary: We have completed proposed research in the First Year Task (i) both antioxidants, N-acetylcysteine and ebselen, overexpression of ROS lowering genes, such as, catalase or PrxIII; and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA by real-time RT-PCR, the rate of DNA synthesis by BrDu incorporation, and different phases of cell cycle by flow cytometry and the most of the part of Task (ii) by determining the morphology and behaviors of cells that over-express mtTRX2, mtSOD, mtPrxIII, catalase or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to estrogen. We previously reported that 17-β-estradiol (E2)-induced mitochondrial (mt) ROS act as signaling molecules. We observed in this study that E2-induced cell growth was reduced by antioxidants N-acetyl-L-cysteine (NAC), catalase, and the glutathione peroxidase mimic ebselen. mtTFA siRNA transfection inhibited estrogen-induced proliferation of MCF-7 cells which is evident from the lower incorporation of BrDU in SiRNA treated cells compared to wild type cells in the presence of E2. We observed similar results by flow cytometery. In E2 treated MCF7 cells, the percentage of DNA content in S phase was 18% while this decreased to 6.8% in mtTFA silenced MCF7 after 24 h (Table I). %. The FACS data not only confirms the results shown by the BrdUrd assay, it also shows that impairment of mitochondrial biogenesis prevents E2-induced entry of MCF7 cells into the S phase by arresting them in the G0/G1 phase. Both antoxidant treatment and dextoxification of ROS prevented E2-induced expression of cyclin D1 and pcna, markers of cell proliferation detected by Real time PCR**.** In cells overexpressed with Adenvirus construct containing catalase that lowers oxidant production as well as in mtTFA silenced cells using their SIRNA, E2 was not able to produce any colony. Both antioxidants ebselen and N-acetylcysteine produced similar effects. It appears that E2 dependent colony formation rate of MCF-7 cells is dependent on ROS or mitochondrial signaling.. These data indicate that E2-induced mtROS are involved in the regulation of early G_1 phase progression and colony formation of breast cancer cells. Since neither antioxidants nor mitochondrial biogenesis blocker used in this study are reported to regulate the ER, our findings suggest that E2-induced mtROS modulates G_1 to S transition and some of the early G1 genes through a nongenomic, ER independent signaling pathway. Thus our results suggest 1) a new paradigm that estrogen-induced mitochondrial oxidants control the early stage of cell cycle progression and colony formation of breast cancer cells, and 2) provide the basis for the discovery of novel antioxidant-based drugs or antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer.

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Appendix

From: Pileggi, Monica J Ms USAMRAA [mailto:monica.pileggi@det.amedd.army.mil]
Sent: Tuesday, February 21, 2006 4:02 PM
To: alvarezp@fiu.edu
Cc: droy@fiu.edu
Subject: BC051097

Dear Ms. Alvarez and Dr. Deodutta,

The following proposal received from your organization for an Idea Award has been forwarded to this office for processing of the award:

"Inhibition of Estrogen-Induced Growth of Breast Cancer by Targeting Mitochondrial Oxidants"

It is requested that the following additional information be provided:

1. Provide your latest Indirect Cost Rate agreement (DHHS agreement).

2. Provide documentation showing the Fringe Benefits Rate Agreement.

3. Verification of salaries requested for the PI and the other personnel. You may do so by providing a copy of a pay stub, Personnel Action Form, or a letter from the institution's Personnel Department stating what the current salary is and for what time period.

4. The Grants Officer Representative has recommended that you revise the budget from two years to three year. This will allow the PI to complete the project in a reasonable time frame.

5. Please revise the travel budget in year two to include $1800 for the Era of Hope meeting.

6. If you are not registered in the Central Contractor Registration (CCR), you are requested to do so immediately. The web site is http://www.ccr.gov and you will need to fill out all information required. This information is necessary in order for our financial office to make payments to your organization once the grant is awarded, so it is extremely important that this information be entered immediately. If your organization is

already registered in the CCR, please verify its accuracy, and insure that your organization status is currently active

7. Your DUNS number is required so that I may get a copy of your latest Representations and Certifications via http://orca.bpn.gov/.

You will have until 24 February 2006 to provide the above information to me so I can make a timely award.

Regards,

Monica J. Pileggi
Contract Specialist
US Army Medical Research Acquisition Activity
USAMRAA-AAA-R
820 Chandler Street
Fort Detrick, MD 21702-5000
(301) 619-2268
(301) 619-3166 (fax)
monica.pileggi@det.amedd.army.mil

From: Kimbark, Donna M Dr USAMRMC
Sent: Friday, January 27, 2006 10:42 AM
To: 'droy@fiu.edu'
Subject: BC051097
Importance: High

Dear Dr Roy,

Congratulations on your Breast Cancer Idea award. As the grants manager for these awards, I will be guiding you through the technical aspects of the award negotiations and the regulatory approval process.

According to your recommended for funding letter all regulatory documents (including animal, human exempt work) were due as of 2 December 2005. At this time none of these documents have been uploaded into your online grant file. Please send in the following documents immediately:

Animal work -
1. IACUC approval
2. PI animal assurances
3. Detailed animal protocol

Human Exempt work -

Claim of Exemption form for all commercially available cell lines

Safety -
https://mrmc.detrick.army.mil/docs/rcq/FY02FSPAppendix.doc
1. Facility safety plan
2. Facility safety manager assurances

Additionally, there were weaknesses in your proposal especially in the research strategy. Please address the issue from your Summary Statement.

One concern is that the proposal does not clearly develop or present the objectives somewhat clear from the technical abstract, some of the descriptions for the experi and methods are confusing. For instance, an inducible lentiviral system is describe suppressing mtTFA in MCF7 cells in culture but the *in vivo* correlate of this work objective does not appear to take this into account. In addition, it would be helpful and control the effects of siRNA against mtTFA to rule out the possibility of off-ta Further, there are several descriptions for monitoring foci formation in the breast c lines utilized, but none of the reported cell lines are appropriate cellular models to induction. Lastly, there is little or no description of pitfalls or alternative strategies proposal.

Please send in all requested documents and information no later than 3 Feb 2006. Further delays will cause delays in obligation of your award to your institute and could jeopardize your funding.

Regards,
Donna M. Kimbark, PhD
Grants Officer's Representative/Grants Manager
Breast Cancer Research Program
IPA of Battelle Memorial Institute in support of
Congressionally Directed Medical Research Program
1077 Patchel Street, Fort Detrick
Frederick, Maryland 21702
Ph. 301-619-6883
FX. 301-619-7796
Donna.Kimbark@amedd.army.mil

From: Droy [mailto:Droy@fiu.edu]
Sent: Friday, February 24, 2006 5:56 PM

To: 'Kimbark, Donna M Dr USAMRMC'
Cc: 'Susan Webster'; 'Dev Pathak'; 'alvarezp@fiu.edu'; 'gutierro@fiu.edu'
Subject: RE: BC051097_the issue from Summary Statement
Importance: High

Dear Dr. Kimabark,
Please find in attach my responses addressing the issue from the Summary Statement. I am not clear whether you would like me to upload this document or address these concerns in the proposal itself. Thanks a lot for your help.
Deodutta

Responses to the weakness identified in the summary statement:

The proposed study plans to examine the highly novel hypothesis that estrogen-induced mitochondrial oxidants are involved in early estrogen-induced signaling that controls the growth of estrogen-dependent breast tumors.

1. We disagree with the reviewer's comment that " there is little or no description of pitfalls or alternative strategies in the proposal". We have proposed to test our postulate using three different alternative strategies, i.e., using a chemical modifier that reduces oxidants, over expressing mt proteins that reduce mt oxidants, and silencing mtTFA that lowers mt oxidant formation by inhibiting mt mass/biogenesis. We have proposed to test over expression of four alternative detoxifying enzymes to lower mitochondrial oxidant formation. These are clearly described in the objectives through out the proposal. The hypothesis will be tested by examining the effects of estrogen-induced reactive oxygen and nitrogen species (RO/NS) on breast tumor cell line growth/transformation properties, determining if overexpression of mitochondrial oxidant-scavenging proteins can inhibit estrogen-mediated proliferation, determining if siRNA-mediated suppression of the mitochondrial transcription factor (mtTFA) can mediate the RO/NS-mediated effects on breast tumor cell line growth/transformation, and determining if chemical modifiers that scavenge RO/NS can inhibit cancer cell growth/transformation.

2. We disagree with the reviewer's comment that "The research design may not distinguish between an observed effect on estrogen-induced tumor cell behavior due to decreased cellular oxidants as proposed and an effect of decreased bioenergetics in the cell with inhibited mitochondrial biogenesis." We have recently published that the concentrations of mitochondrial inhibitors that do not alter the ATP levels inhibit estrogen-induced DNA synthesis and -growth of MCF-7 cells (Oncogene, 2005, 24(31):4883-93).

3. The concern about the availability of STZN: We have just learnt that stilbazulenyl nitrone (STAZN) will be not available for this project, because this compound is not synthesized any more by Dr. David Becker's laboratory nor it is a commercially available. Instead of STZN as a chemical modifier of RO/NS, we propose to use antioxidants, N-acetylcysteine and ebselen as modifiers of RO/NS. This will not impact the testing of the proposed concept, because (i) we have recently shown that (Oncogene, 2005,24(31):4883-93; Biochemistry, 2005, 44(18):6900-6009) that antioxidants, N-acetylcysteine and ebselen, are very effective in preventing estrogen-induced transition from G1 to S phase; (ii) These antioxidants inhibit estrogen-induced activation of transcription factors and cell cycle gene (Oncogene, 2005, 24(31):4883-93); and (iii) instead of

STZN, now this will be accomplished by using N-acetylcysteine and ebselen. We have replaced STZN with antioxidants N-acetylcysteine and ebselen in the objective of this proposed study. **Please see below the revised technical abstract objectives in this section and research strategy in the next section showing changes highlighted with underlined and bold.**

Revised Objective 1: To determine whether estrogen-induced mtRO/NS are involved in estrogen-induced in vivo growth of malignant breast epithelial cells. To determine this we will examine whether overexpresion of mt superoxide dismutase (SOD) or catalase that detoxify ROS; or co-treatment with the chemical antioxidant modifiers, **N-acetylcysteine and ebselen** that reduces oxidants, prevents estrogen-induced cell transformation and in vivo growth of malignant breast epithelial cells. Objective 2: To determine whether over-expression of the mitochondrial oxidant scavenging proteins mt thioredoxin 2 (TRX2) or mt peroxiredoxin III (PrxIII), is involved in estrogen-induced cell transformation and in vivo growth of malignant breast epithelial cells. Objective 3: To determine whether knockdown of mitochondrial biogenesis through silencing of mtTFA that will lead to the attenuation of RO/NS production prevents estrogen-induced cell transformation and in vivo growth of malignant breast epithelial cells.

4. Based on the reviewer indication that the technical abstract describes clearly the research strategy, the description for the experimental design and methods. We will use the same contents from the technical abstract to address the concerns about research strategy, experimental designs and methods.

In all three objectives, we will measure the rate of growth, transforming activity, angiogenesis and invasiveness. (i) We will demonstrate that the antioxidants **N-acetylcysteine and ebselen;** overexpression of mtTrx2, PrxIII, catalase, or mtSOD; and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA by real-time PCR and western blotting; the rate of DNA synthesis by BrDu incorporation, and different phases of cell cycle by flow cytometry in estrogen-free medium, (ii) We will also examine the morphology, apoptosis and behaviors of cells that over-express mtTRX2, mtSOD, mtPrxIII, catalase; or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to various concentrations of estrogen. (iii) Whether estrogen-induced conversion of normal cells to transformed cells is inhibited by treatment with **N-acetylcysteine and ebselen**; overexpression of MnSOD, catalase, PrxIII, Trx2, or mtTFA silencing. Cell transformation will be determined by: (a) Foci Formation, (b) Anchorage-independent (soft agar) cell growth, and (c) tumor spheroid formation using new 3D HuBiogel bioassay. (iv). We will use a novel physiologically-relevant 3D human biomatrix, HuBiogel™ assay as well as nude mice xenograft model to determine whether co-treatment with **N-acetylcysteine and ebselen**; overexpression of MnSOD, catalase, PrxIII, or Trx2; and silencing of mtTFA in the breast cancer cells following exposure to estrogen prevents growth, angiogenesis, and invasion of tumors. We will test silencing effects of mtTFA on in vivo tumor growth and we will use controls to rule out any effects of inducer system (using theconstruct without mtTFA insert and scrambled mtTFA SiRNA) or inducer (antibiotics).

5. Significance of the proposed study will not change with substituting STZN with N-acerylscysteine and ebselen. From the Technical Abstract: “The novel finding which will emerge

from this study is that estrogen-mediated regulation of transcription of early G1 genes is through RO/NS produced by mitochondrial biogenesis and will have major implications in understanding the role of estrogen in the progression of breast cancer cells. This will not only provide a new paradigm in understanding the mechanism of estrogen-induced proliferation, but will also validate the novel concept by which estrogen-induced mitochondrial oxidants signal, and this, in turn, will provide information for the design of new anti-oxidant-based drugs or new antioxidant gene therapy target for the prevention and treatment of breast cancer."